Candidate drugs binding the Anaphase Promoting Complex: A novel target for anti-cancer therapy

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Abstract

Spindle poisons (e.g. paclitaxel) induce mitotic arrest and apoptosis in cancer cells. The anaphase promoting complex/cyclosome (APCC/C) is an E3 ubiquitin ligase and the master regulator of cell cycle progression. Activation of the spindle assembly checkpoint (SAC) by spindle poisons inhibits the APC/C and induces mitotic arrest. Spindle poisons depend on functional SAC to induce mitotic arrest. Direct inhibition of the APC/C obviates the need for a functional SAC. Homology structure models for interacting APC/C subunits ANAPC11 and ANAPC2 were used for in silico screening of ANAPC2 to identify compounds predicted to interfere with ANAPC11 binding. Previous studies showed 3 compounds induced mitotic arrest and apoptosis in cancer cells but not in telomerase immortalized human fibroblasts. Thermal denaturation assay of recombinant protein can confirm on-target binding. Expressing recombinant 6XHis tagged ANAPC2 failed. The ANAPC2 caspase was then ligated into a new expression system utilizing a chitin-binding affinity tag and intact mediated purification. C-terminal (pTXB10) and X-terminal (pTXB21) fusion constructs were prepared and tested in three expression hosts and evaluated for optimal yield. Large scale purification of recombinant ANAPC2 using E. coli host BL21 expressing the N-terminal fusion protein is in progress. Compound binding to purified recombinant ANAPC2 will be determined. Compound binding of ANAPC2 will indicate that compounds targeting the APC/C can induce mitotic arrest and kill cancer cells while sparing normal cells and may be an effective approach to developing new anti-cancer drugs. Supported by National Cancer Institute grant R01 CA-134293.

Introduction

Cancer, a disease of uncontrolled cell proliferation, is the second leading cause of death in the U.S., killing over 1.5 million people a day. Worldwide cancer deaths reached 7.6 million in 2008. Although it is lower on the list of causes of death in developing countries, cancer rates have slowly risen as the life expectancy increases.

In the US, survival rates have improved largely due to better detection and early treatment of the disease, not because of new chemotherapeutics. However, a growing number of patients are encountering cancers that simply can’t be eradicated with the traditional chemotherapeutics.

Paclitaxel, a widely used drug for several types of cancer, disrupts mitotic spindle function through tubulin stabilization. The use of treatment relies on a functional spindle assembly checkpoint (SAC) that can signal the cell to pause in mitosis due to increased alignment tension of sister chromatids at the metaphase plate. One function of the SAC is to inhibit the anaphase promoting complex/cyclosome (APCC/C) which is the master regulator of mitotic progression.

Many cancers resistant to paclitaxel lack a functional SAC. Other cancer cells (A2780/CP70) were treated with the IC50 for 48 hours. Paclitaxel was used as a positive control. Slides were prepared for mitotic index and mitotic catastrophe determination. All compounds induced increases in mitotic index in ovarian cancer and diploid fibroblasts. (*p < 0.05).

Anaphase Promoting Complex

- An E3 Ubiquitin Ligase, responsible for the transfer of ubiquitin molecules to a protein target on a susceptible histone or non-histone protein via a histone or non-histone protein.
- Components of ten subunits and two potential activating proteins.
- Catalytic subunit ANAPC11 (a zinc RING finger protein), Scaffolding subunit ANAPC12 (a ubiquitin-like protein), and Activator proteins CDCC2 or CBD1 are responsible for selective enzymatic function.
- Master regulators of mitosis and targets include securin, Cyclin B, Cyclin C, A, geminin and many others.
- Target protein ubiquitination results in degradation by the 26S proteasome.
- Securin degradation releases securin from inhibition and allows it to cleave cohesin. Cohesin acts like a ‘glue’ to hold sister chromatids together.

Hypothesis

If candidate compounds block the binding of subunits of the APC/C and inhibit it, then cancer cells will undergo mitotic arrest and either undergo apoptosis during mitosis or proceed to arrest and undergo apoptosis in early G1.

Methods

Figure 1. A simplified model of the interaction between the spindle checkpoint and the APC/C during mitosis.

A. In late prophase, the CDC20 co-activator of the APC/C is sequestered by the spindle assembly checkpoint. Pairs of sister chromatids are held together by cohesion. Separase enzyme is inhibited by securin.

B. In mitosis, microtubules reorganize and the CDC20 co-activator is released. CDC20 binds to the APC/C, enabling it to target securin for degradation. Separase enzyme is activated. Compound binding of ANAPC2 would inhibit the APC/C, thus separase would remain inactive.

C. In M phase, the active separase enzyme cleaves the cohesin protein. This allows separation of the sister chromatids, which begins to move toward polar regions of the dividing cell.

Figure 2. Mitotic index determination. Ovarian cancer cells (A2780/CP70) were treated at the IC50 for compounds 6, 10, and 11 for 24 h. Fibroblasts (IMGM4) were treated for 48 h. Paclitaxel was used as a positive control. Slides were prepared for mitotic index and mitotic catastrophe determination. All compounds induced increases in mitotic index in ovarian cancer and diploid fibroblasts. (*p < 0.05).

Figure 3. Assessment of caspase 3 activity. Ovarian cancer cells (A2780/CP70) were treated at the IC50 for 24 h. Caspase 3 activity is measured using the EnzCheck™ Caspase 3 Assay Kit. Enzyme activity is calculated using the IC50 for each compound.

Figure 4. Cloning Strategy. A. The ANAPC2 cassette is transferred from the pQE30 plasmid utilizing a KRX (KRX = KRX) vector into pTYB10 and pTYB21 plasmids that utilize an internal sap tag, pTYB10 uses restriction site for Nde1 and Xho1; pTYB21 uses restriction sites for Nde1 and Sac1.

B. The fusion protein contains a self-cleaving intein tag and a chitin binding domain. The fusion protein will bind to a chitin column and cleaved intein protein collected in fractions and transferred to storage buffer.

Future Directions

Figure 5. DNA amplification and plasmid construction.

Candidate drugs binding the ANAPC2 cassette with primers containing restriction sites for new expression system. Sample 1, Nde1; Sample 2, Nde1 + Sac1; Sample 3, Nde1 + Xho1; Sample 4, Xho1 + Sac1.

Figure 6. Expression optimization.

Three E.coli expressions hosts (S3580, BL21, T70: data not shown) were transformed with plasmids containing C-terminal (X-terminal and Y-terminal) fusions of the ANAPC2 cassette. Transformed colonies inoculated at 37°C and expression was induced by addition of 0.4 mM IPTG. Samples collected every hour to monitor expression. Immunodetection of CBD expressed right. Fusion protein is ~95 kDa. ANAPC2 is ~40 kDa.

Figure 7. Protein purification and screening.

Purified ANAPC2 was tested by thermofluor assay in order to confirm on-target binding of candidate compounds. Western blot analysis was applied to the purified protein in a 9% SDS-PAGE gel and transferred to the membrane.

Conclusions

- Compounds targeting the APC/C induced mitotic arrest in cancer cells and diploid fibroblasts. Activation of caspase 3 in cancer cell lines but not diploid fibroblasts suggests candidate compounds may be developable into cancer-selective drugs.

- The ANAPC2 cassette is successfully ligated into an expression system that expressed an appreciable amount of protein. After purification refinement, a binding study will be viable.

- On-target binding can be determined through high-throughput thermofluor screening. This protocol provides a means of testing more candidate compounds in our catalog. If inhibition of APC/C function is verified, the APC/C may be a promising target for future chemotherapeutics.

Acknowledgements

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Figure 8. Thermostable assay

Purified ANAPC2 will be tested by thermostable assay in order to confirm on-target binding of candidate compounds. Fluorescent dye is added to the purified protein in a 9% well plate and heated.

As the protein the unfolds, the dye binds to the hydrophobic regions of the protein and becomes intensely fluorescent. Definitive shifts in the melting curve will confirm binding.


References

Endocytic Trafficking of Mutant Epidermal Growth Factor Receptors in Lung Cancer
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Abstract

Purpose: To better understand the endocytic pathway and mechanism of how activating T790M mutants of epidermal growth factor receptor (EGFR) regulates signaling in two lung cancer cell lines: PC-9 and H1650.

Methods: PC-9 and H1650 cells express mutant EGFRs; HeLa cells express wild type EGFRs. Immunoblotting was used to test for time-dependent EGFR phosphorylation and degradation. Receptor trafficking and endosomal accumulation was observed by immunofluorescence staining of the EGFR. Kinetics of 125I-EGF endocytosis and ligand-mediated degradation was measured by radioligand binding assay.

Results: Immunoblotting shows that EGFR treatment causes an increase in the basal activity of EGFR phosphorylation, and peaks at 30 minutes. Immunofluorescent staining of the EGFR indicates that a substantial amount of the unliganded, mutant EGFR accumulates in the early endosome and EGF treatment further increases endosomal accumulation in the mutant EGFR. Furthermore, there is a slower rate of receptor degradation of the mutant EGFRs compared to wild type receptor, as seen in the radioligand binding assay.

Conclusions: Activating mutants of EGFRs found in lung cancer cells not only have higher basal phosphorylation, but also defective endocytic trafficking. Based on radioligand binding and immunofluorescence experiments, it can be concluded that the receptor trafficking defect causes increased receptor accumulation in the endosome. Less degradation in the cancer cell lines indicates there is a disruption in the endocytic trafficking of mutant EGFRs.

Introduction

• Lung cancer is a major health concern in the Commonwealth of Kentucky, and there are more deaths in the U.S. from lung cancer than any other cancer. (www.cdc.gov)
• Specifically, over 85% of lung cancers are non-small cell lung cancer (NSCLC), and these cells are used as a model in this project. (www.cancer.org)
• Many NSCLC cells are characterized by activating EGFR mutations.
• EGFR is a receptor tyrosine kinase on the cell surface that is normally activated by ligand binding, and inactivated by either endocytic recycling or degradation.
• Common Mutations found in studies:
  - EGFR V895L, T790M
  - Erlotinib and gefitinib: EGFR inhibitors

Hypothesis

Constitutively active epidermal growth factors have altered endocytic trafficking.

Figure 1

EGFR Phosphorylation and Degradation in PC-9, H1650, and HeLa cells

Figure 2

Immunofluorescence Images of PC-9, H1650, and HeLa cells

Figure 3

125I-EGF Binding Assay

Figure 4

Model of T790M EGFR Endocytic Trafficking

Conclusions

• Activating mutants of EGFRs in lung cancer cells result in a receptor that is constitutively active, as indicated by the partial phosphorylation.
• There is substantial intracellular accumulation of unliganded EGFRs in lung cancers.
• Addition of ligand further increases receptor accumulation in the endosome.
• Less degradation in the cancer cell lines indicates there is a disruption in the endocytic trafficking of mutant EGFRs.

Future Experiments

- Determine where T790M localization is occurring, and what effector molecules are allowing sustained activities of EGFR.
- Determine how EGFR activities are related to downstream signaling.

Acknowledgements

R25 Cancer Education Program, Joanne Peterson, Jamie Rush, Eileen Parks, Nicole Jackson; Funding: NIH/NCI R25-CA134283.
Introduction

In sequences of nucleic acid with particularly high proportions of guanine, four guanine bases can assemble themselves into a planar, four-stranded, Hoogsteen hydrogen-bonded structure. A single strand of DNA is known as a G-tetrad. However, multiple G-tetrads can stack on each other to adopt various loop configurations - producing a structure called a G-quadruplex. Though their full function is not completely understood, their high level of polymorphism and presence in critical progression of this project was as follows:

1. Experimental coordinate values of a known quadruplex were taken and used to generate a starting model [2].
2. A survey of torsion angles and bond distances was taken from known quadruplexes TGGGGAGGG and TGGGGATGGG, using the JmolView utility (Jmol). These torsions were used throughout the project to determine whether our artificially manipulated structures fell within acceptable ranges of known values.
3. The nucleic acid builder (nab) and Perl languages were used to write programs that could alter the various properties that differentiate between quadruplexes (such as sugar conformation, stacking order, etc.)
4. The Perl script Quadgen was developed in order to choreograph these various nab programs and Perl subroutines; its job is to take two reference tetrads and develop them into the full set of possible quadruplexes.
5. The structures produced by Quadgen were minimized in order to find their more low-energy forms.
6. Similar to step 2, a survey of torsion angles was taken from the loops of experimental quadruplexes.

The flow of this project revolved around a two-step process of first manually effecting a change in a reference structure, then writing a computer script that would automate the process. The stepwise progression of this project was as follows:

Methods

The flow of this project revolved around a two-step process of first manually effecting a change in a reference structure, then writing a computer script that would automate the process. The stepwise progression of this project was as follows:

1. Take in a number of tetrads per quadruplex as a user-defined argument.
2. Take in a list of loop shapes.
3. Generate list of all possible stacking orders.
4. change_sugar.nab
   - Adjusts ch2 torsion to either anti or syn conformation.
   - Applies conformation to sugar.
5. adjust_dihedrals.nab
   - Adjusts beta, gamma, and possible torsion.
   - Takes in name of strand.
   - Takes in name of strand.
6. stack_tetrads.nab
   - Rotates and translates tetrads to form quadruplex structures.
   - Takes specific tetrads.
   - Calculates rotation needed.
7. &assemble_residues.nab
   - Takes in name of quadruplex.
   - Takes stranded fragment.
   - Fixes atom order and numbering.
8. bend_loops.nab
   - Rotates local residue to have the desired angle.
9. superimpose.nab
   - Fits the duplicated loop into proper position on strand.
10. &add_potassium
    - Adds potassium.

Quadgen Program Flow

Generated table of possible sugar configurations (e.g. anti, anti, syn, syn)
Names of the 2 tetrads per stacking group e.g. TGG, TGG, TGG
Number of tetrads per quadruplex
Variations with repetition subroutine
   - Take in a list L
   - Take in a number N
   - Output all permutations of L from 1 to N
List of all possible tetrads stacking orders
change_sugar.nab
   - Adjusts ch2 torsion to either anti or syn conformation.
   - Applies conformation to sugar.
16 unique tetrads with varying sugar conformations
16 adjusted tetrads ready to stack
stack_tetrads.nab
   - Rotates and translates tetrads to form quadruplex structures.
   - Takes specific tetrads.
   - Calculates rotation needed.
   - Generates a transformation matrix
   - Transforms tetrads using matrix.
   - Repeats from beginning
16 adjusted tetrads ready to stack
16 unique tetrads with varying sugar conformations
16 adjusted tetrads ready to stack
16 unique tetrads with varying sugar conformations
16 adjusted tetrads ready to stack
16 unique tetrads with varying sugar conformations
16 adjusted tetrads ready to stack

Sample Execution

Sequence

Possible quadruplexes (1D) [39 possible structures]

Possible quadruplexes (2D) [57 possible structures]

Possible quadruplexes (3D) [1 possible structure]

References

1. James Graham Brown Cancer Center, Department of Biochemistry and Molecular Biology, Department of Medicine

Conclusions

The programs were tested using the human c-myc nucleoside sequence, and in order of minimization, the set of quadruplex models appeared to be structurally accurate and exhibit the full range of quadruplex polymorphism. Despite structural accuracy, the relevance of many of these quadruplexes is still unknown. It is, however, to examine the biophysical properties of these structures to determine if they exhibit those attributes that would make them valuable as possible molecular targets.

Acknowledgments

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In vivo and in vitro determinates that regulate BCLxl’s apoptotic potency

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Abstract

BCLxl, a member of the B-cell lymphoma-2 (BCL-2) family, is a protein that plays a key role in cell survival by preventing mitochondrial outer membrane permeabilization (MOMP). The ability of BCLxl to block apoptotic signals in the cell has been positively linked to tumorigenesis. Previous in vivo experiments in the lab using mouse models showed the significance of individual domains within BCLxl’s protein structure for dictating oncogenic potency. Specifically, the Bcl-2 homology (BH)-4 domain of BCLxl has been shown to be essential in the anti-apoptotic functionality of BCLxl. In vitro experiments were performed to analyze how altering the protein structure of BCLxl affected protein stability. Cells were transfected with chimeric BCLxl proteins and then treated with either a proteasome inhibitor (MG132) or a protein biosynthesis inhibitor (cycloheximide). Data showed that cells treated with cycloheximide significantly decreased the expression of BCLxl, whereas cells treated with MG132 had no change or an increase in levels of protein expression. In contrast, treatment of chimeric BCLxl protein expression increased dramatically within 4 hours of MG132 treatment, but by 16 hours post-treatment, levels had decreased and returned to levels seen pre-treatment. Previous research has shown certain residues in the BH4 domain of Bcl-2 to be significant to the potency of the protein. Attempts were made to induce point mutations in the BH4 domain of BCLxl through site-directed mutagenesis. The data obtained from these analyses will be used to standardize results of future experiments.

Results

Figure 1. In vivo apoptotic pathway: directed by Bcl-2 protein mediation of mitochondrial outer membrane permeabilization.

Figure 2. Structures of BCLxl and BCLb and chimeric constructs. Construct 5 includes the BH4 and loop domains of BCLxl; construct 7 only includes the loop domain of BCLxl.

Figure 3. In vivo BCLxl/BCLb chimeric construct survival curves showing percent survival post transplant for mice infected with BCLxl to be similar to those infected with construct #5, and separately, percent survival for those infected with BCLxl similar to those infected with construct #7.

Figure 4. Percent survival post transplant for mice infected with BCLxl constructs with altered transmembrane domains. Percent survival of BCLxl and BCLxl cb5 are similar. BCLxl ActA shows a slight increase in survival.

Figure 5. Western blot of in vivo BCLxl constructs. BCLxl, BCLxl cb5, and BCLxl ActA show a significant increase in levels of protein expression compared to wild type mice and those infected with BCLxl GFP or BCLbs.

In vivo expression of BCLxl constructs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BCLxl</th>
<th>BCLxl cb5</th>
<th>BCLxl ActA</th>
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<td>BCLxl cb5</td>
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<tr>
<td>BCLxl ActA</td>
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</table>

Conclusions

The various regulators of BCLxl’s potency has been explored in both in vivo and in vitro environments. It can be seen that sequestering the protein to either the mitochondria or the endoplasmic reticulum through transmembrane domain swaps does not significantly affect the potency of BCLxl. Treatment with the protein biosynthesis inhibitor cycloheximide decreases protein expression in stability tests but this was not visible in the time course experiment. Treatment with the proteasome inhibitor MG132 results in a stabilization in protein levels for constructs 5 and 7 but the same result is not seen in BCLxl. Point mutations via site directed mutagenesis will be utilized in future experiments to further examine the potency of mutated BCLxl.

Acknowledgements

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Colorectal cancer (CRC) is the third most common cancer worldwide. Early detection is key in successful CRC treatment.

Dysregulated miRNA expression is known to play a role in carcinogenesis.

Protein targets of miRNAs are potential therapeutic modalities. PDCD4, PTEN, and TGF-β are protein targets of miRNA 21.

RASA1 is a protein target of miRNA 31.

Previous results indicate miRNA expression in snap-frozen colon tissue significantly differs between non-neoplastic and cancerous samples.

Formalin fixed paraffin embedded (FFPE) tissue is ideal for experimental use because it can be stored for an extended period of time.

Laser capture microdissection (LCM) is a technique that allows for the isolation of specific tissue sections and cell populations.

Similar miRNAs will be significantly dysregulated in both fresh and FFPE (formalin fixed paraffin embedded) CRC tissue.

Proteins targets of dysregulated miRNAs will have different expression levels in CRC and normal tissue.

Cut serial sections of FFPE tissue

Laser Capture Microdissection (LCM)

miRNA Isolation

qRT-PCR

Protein Extraction

Western Blot

Tissue from colon cancer patients was excised, fixed in formalin and embedded in paraffin. Serial sections (7 µm) were cut from FFPE tissue. Non-neoplastic and cancerous tissue was isolated from FFPE samples via LCM. miRNA was isolated from tissue. cDNA synthesis, pre-AMP, and qRT-PCR were performed. Protein was extracted from remaining FFPE tissue for western blot analysis.

Introduction

Results

qRT-PCR

A. Representative western blot of PDCD4, PTEN, RASA1 and TGF-β in colon tumors and nonneoplastic tissue from four patients. B. Densitometry analysis of PDCD4, PTEN, RASA1 and TGF-β normalized to β-actin control. Protein extracts were prepared and 100 µg of tissue was loaded per lane on an SDS-PAGE.

Key Findings

Preliminary qRT-PCR data indicated that miR-31 and miR-21 were most dysregulated (upregulated) in cancer.

Protein extraction from FFPE tissue resulted in low yields.

Western blot results inconclusive.

Conclusions and Future Directions

Slight differences in fold regulation between FFPE and snap frozen tissue may be due to miRNAs present in stroma.

Future protein analysis:

- Optimize protein extraction from FFPE tissue
- Use samples with same histological grade for western blot
- Explore additional tumor suppressor pathways that may be affected by dysregulation of miRNAs.

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